Confirmation No.: 1008

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No	:	09/643,755
Applicant	:	Gijs van Rooijen, Richard Glenn Keon, Yin Shen and Joseph Boothe
Filed	:	August 23, 2000
Title	:	Commercial Production of Chymosin in Plants
TC./A.U.	:	1638
Examiner	:	Georgia L. Helmer
Docket No.	:	9369-153
Customer No.	:	001059

Honorable Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313–1450

DECLARATION UNDER 37 C.F.R. 1.132

Honourable Assistant Commissioner
For Patents
Washington, D.C. 20231

Dear Sir:

I, W. Brent R. Pollock, citizen of Canada and resident of Calgary, Alberta, Canada, declare that the following facts are within my knowledge and are true.

1. I am the Group Leader of Process Development at SemBioSys Genetics Inc. I attach a copy of my curriculum vitae as Exhibit A.

2. I have reviewed the Official Action for the application that issued on September 27, 2006.

3. Experiments have been conducted by me which demonstrated that employing a hexane extraction method destroys the activity of chymosin. In

contrast, extraction in an aqueous buffer as described in the present application does not destroy the activity of chymosin. Details of the experiment are summarized below.

4. Procedure:

Seed-Free Control:

0.2 mL of SPC (Seed-Produced Chymosin) reference lot HIC021018F5 was added to 1.0 mL of hexane:2-PrOH (3:2 v/v) and mixed for 1' on high using a vortex mixer. The mixture was centrifuged briefly and the upper (organic) phase was removed; it was noted that a white precipitate was in the tube, which was assumed to be denatured chymosin. Chymosin concentration in the aqueous phase was assayed by milk clotting activity compared to the unextracted reference material.

Extraction with Buffer:

0.5 g of SPC seed PTS ID Cen-IH-23-2165-15 was added to 2.5 mL of extraction buffer (2.0 M NaCl, 0.05 M NaH_2PO_4, pH 5.9 w/NaOH) in a mortar and ground for 1' with a pestle. The slurry was distributed between two 1.5 mL microfuge tubes and spun at 14,000 rpm for 11' in the Eppendorf 5417 centrifuge at ambient lab temperature (19-23°C). The aqueous phase was removed using a syringe and needle, then clarified through a 0.2 micron filter (Pall #4192). Chymosin concentration in the clarified aqueous phase was assayed by milk clotting activity compared to the reference lot.

Extraction with Hexane:

0.5 g of SPC seed PTS ID Cen-IH-23-2165-15 was added to 2.5 mL of hexane:2-PrOH (3:2 v/v) in a mortar and ground for 1' with a pestle. The fluid was removed by decanting. Extraction & decanting were repeated twice more. Then a 2.5 mL aliquot of 2.0 M NaCl, 0.05 M NaH_2PO_4, pH 5.9 w/NaOH was

added to the defatted grounds and pestlated for another 1'. Using the same procedure noted for the extraction with buffer, the slurry was centrifuged, sampled, filtered and assayed for chymosin activity.

5. Results:

As demonstrated in Figure 1, which is attached as Exhibit B, the extracted chymosin lot lost all milk clotting activity after being subjected to extraction with hexane:2-PrOH. In contrast, the extracted chymosin showed 100% of the expected activity when extracted directly with the aqueous buffer normally used in Applicant's method. When this seed lot was instead extracted with hexane:2-PrOH prior to the normal aqueous extraction, it only showed 31% of the activity detected in the positive control extract.

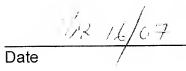
6, Experiments have also been conducted by me which demonstrate that dry crushing of seed prior to protein extraction, destroys the activity of chymosin. Details of the experiments are provided below.

7. Initial cracking of 22 kg of SPC Safflower seed was performed using a Cold Press. The cake was stored as dry fingers; both the oil and cake fractions were placed in the cold room. Mortar and Pestle grinding of a 0.5 g sample of the cake showed no milk clotting activity extractable by 0.05 M NaH_2PO_4, pH 5.9, w/0.35 or 1.0 M NaCl; the positive (SPC) and negative (WT) seeds tested as expected. See Figure 2 which is attached as Exhibit C. BSA indicates that general protein extraction was not the problem. Therefore, protein was extracted but was not active. See Figure 3 which is attached as Exhibit D.

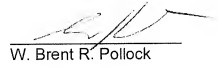
8. Therefore, the above experiment demonstrates that extraction of chymosin from seed using hexane extraction and/or dry crushing destroys the

activity of the chymosin and therefore such a procedure would not be used in a manufacturing method for the isolation of chymosin in seed.

9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and, further, that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such a wilful false statement may jeopardize the validity of the application or any patent issuing thereon.



Date



W. Brent R. Pollock

EXHIBIT A

Curriculum Vitae

Dr. W. Brent R. Pollock

1239 Mackid Rd NE
Calgary, Alberta, Canada
T2E 6A8
Home Phone: 1-403-230-4346
E-mail: b3b@telusplanet.net

CITIZENSHIP:
Canadian

WORK EXPERIENCE:

Purification of Proteins & Peptides:

- purification at milligram- through kilogram-scale under GMP guidelines
- recovered from recombinant and native hosts grown using bacterial fermentation, mammalian cell culture or Safflower seed apoplast and oilbodies
- scalable parameters (*e.g.* bed height, linear velocity, flux, mixing times, mass transfer times, mass load)
- operational costs (*e.g.* hardware, raw material cost and availability, throughput in total time and %/hour/person);
- operations:
  - solvent selection and preparation (L- to kL-scale)
  - centrifugation (batch & continuous flow)
  - microfiltration (clarification & sterilisation)
  - ultrafiltration (centrifugal, stirred-cell, TFF from mL- to kL-scale)
  - virus removal by nanofiltration or pH control
  - preparative medium & high pressure column chromatographies using GE Healthcare UNICORN controllers (column i.d. 0.1 to 20 cm):
    - Protein A Affinity
    - IEC
    - HIC
    - SEC
    - RPC
    - R-D-RPC
  - preparative RP-HPLC
- analysis of purity and impurity:
  - UV-Visible Spectrophotometry
  - SE-HPLC-UV-Vis & RP-HPLC-UV-Vis
  - Peptide Mapping
  - Mass Spectrometry
  - N-terminal sequencing
  - Amino Acid Analysis
  - IEF
  - SDS-PAGE
  - Immunoblotting
  - Fluorescence (Internal and External Fluorophores)
  - Right Angle Light Scattering
  - NMR
  - Circular Dichroism
  - Host Cell Proteins
  - Endotoxin (LAL)
  - DNA

- GC of residual organic solvents
- HPLC of simple carbohydrates
- Bioassay
- Viral assay

Administrative & Regulatory

- modifying the Purification Development group at Biomira and improving electronic record keeping for tracking and summarising process parameters and performance
- establishing the Downstream lab in the Bioprocess Division at Altarex which entailed making purchasing recommendations (*e.g.* preparative chromatography controller; pH/conductivity meter & probes; HPLC controller & columns; CE controller; slab gel system; stock chemicals; balances; calibration standards; sample handling & storage system; micro, ultra- & nanofiltration systems)
- technology transfer to contract GMP manufacturing site and Man-in-Plant during production of GMP materials
- initiation and coordination of research activities performed by a Contract Research Organisation
- interviewing/training/managing personnel
- communicating with clients/contractors/vendors
- single project budgeting for a group of three to five personnel
- contributing to the CMC section of an IND submission
- proposal for an 18-month strategic research grant
- technical section of an equivalency study
- familiarity with USFDA and ICH guidelines for production of products derived from recombinant organisms
- preparation and use of controlled documentation (*e.g.* SOPs, BPRs, CFs & LUMACs)
- comprehensive reports on development and production activities

Other technical knowledge (practical and theoretical):

- gene cloning & sequencing (eukaryotic and bacterial)
- design, construction and optimisation of recombinant protein expression systems
- mechanisms of genetic transfer, structure & regulation
- protein (especially metalloprotein) synthesis, post-translational modification & translocation
- electron transfer mechanisms & pathways
- transmembrane protein complexes
- haem biosynthesis
- aerobic vs. anaerobic metabolism
- radionuclide labelling

WORK HISTORY:

Jan. 2007 – Present:	promoted to Group Leader, Process Development
March 2002 – Dec. 2006:	Protein Purification Scientist in charge of purification process development at SemBioSys Genetics, Inc.
Jan. 1999 - Nov. 2001:	Senior Scientist managing the Purification Development Group at Biomira, Inc.
Dec. 1997 - Dec. 1998:	Purification Process Specialist at AltaRex Corp.
April 1997 - Oct. 1997:	Senior Protein Biochemist at INTELLIgene Expressions, Inc.
July 1995 - March 1997:	Research Associate for Nobel Laureate Dr. Michael Smith in the Biotechnology Laboratory at the Univ. of British Columbia. Project - Cloning, sequencing & recombinant expression in *E. coli* & *P. pastoris* of laccases from the white rot fungus *Trametes versicolor*.
Aug. 1992 - June 1995:	Post Doctoral Fellow with Prof. Grant Mauk in the Faculty of Medicine at the Univ. of British Columbia. Project - Expression, purification & biophysical analysis of recombinant eukaryotic cytochromes produced in *E. coli*.

PROFESSIONAL MEMBERSHIPS:

2000 – ongoing:	American Chemical Society
2000 – 2001:	Parenteral Drug Association
1990 – 1998:	American Society for Microbiology

POST-SECONDARY EDUCATION:

Nov. 1992:	Awarded the Degree of Doctor of Philosophy (Biochemistry)
June 1992:	Defended Ph. D. Thesis (Molecular biology of *c*-type cytochromes from the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough)

Sept. 1987 - July 1992:	The University of Calgary (Graduate Studies)
June 1987:	Awarded the Degree of Bachelor of Science (Honours) Major: Biochemistry Minor: Chemistry
Sept. 1983 - June 1987:	The University of Calgary (Undergraduate)

REFERENCE CONTACTS:

1. **Laura Pflug**, Director of Product Development, Biomira U.S.A., 1002 East Park Blvd., Cranbury, NJ 08512, U.S.A. *Ph: 1-609-655-5300 x.236.* E-mail: lpflug@biomira.com
2. **Dr. Tom Facklam**, Vice President of Technical Operations and Quality, Biomira, Inc., 2011 - 94 St., Edmonton, Alberta, CANADA T6N 1H1. *Ph: 1-780-450-3761 x.217.* E-mail: tfacklam@biomira.com
3. **James Tasker**, Director of Finished Product Operations, AltaRex US Corp., 610 Lincoln Street, Waltham, MA, 02451 U.S.A. *Ph: 1-888-801-6665 x.1514.* E-mail: jtasker@altarex.com
4. **Dr. A. Grant Mauk**, Department of Biochemistry & Molecular Biology, The University of British Columbia, Vancouver, British Columbia, CANADA V6T 1Z3. *Ph: 1-604-822-3719.* E-mail: mauk@unixg.ubc.ca
5. **Dr. Gerrit Voordouw**, Division of Biochemistry, Department of Biological Sciences, The University of Calgary, Calgary, Alberta, CANADA T2N 1N4. *Ph: 1-403-220-6388.* E-mail: voordouw@acs.ucalgary.ca

PUBLICATIONS:

Published journal papers:

1. Pollock, W.B.R., Rosell, F.I., Twitchett, M.B., Dumont, M.E., & A.G. Mauk (1998). Bacterial expression of a mitochondrial cytochrome *c*. Trimethylation of Lys72 in yeast *iso*-1-cytochrome *c* and the alkaline conformational transition. *Biochemistry.* **37**(17): 6124-6131.
2. Ong, E., Pollock, W.B.R., & M. Smith (1997). Cloning and sequence analysis of two laccase complementary DNAs from the ligninolytic basidiomycete *Trametes versicolor*. *Gene*. **196**(1-2): 113-119.
3. Pollock, W.B.R. & G. Voordouw. (1994) Molecular biology of *c*-type cytochromes from *Desulfovibrio vulgaris* Hildenborough. *Biochimie* **76**: 554-560.
4. Pollock, W.B.R. & G. Voordouw. (1994). Aerobic expression of the *cyf* gene encoding cytochrome *c*-553 from *Desulfovibrio vulgaris* Hildenborough in *Escherichia coli*. *Microbiology* **140**: 879-887.
5. Blanchard, L., D. Marion, B. Pollock, G. Voordouw, J. Wall, M. Bruschi, & F. Guerlesquin. (1993). Overexpression of *Desulfovibrio vulgaris* Hildenborough cytochrome c_{553} in *Desulfovibrio desulfuricans* G200: evidence of conformational heterogeneity in the oxidized protein by NMR. *Eur. J. Biochem.* **218**: 293-301.
6. Rossi, M., W.B.R. Pollock, M.W. Reij, R.G. Keon, R. Fu, & G. Voordouw. (1993). The *hmc*-operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough encodes a potential transmembrane redox protein complex. *J. Bacteriol.* **175**: 4699-4711.
7. Bruschi, M., P. Bertrand, C. More, G. Leroy, J. Bonicel, J. Haladjian, G. Chottard, W.B.R. Pollock, & G. Voordouw. (1992). Biochemical and spectroscopic characterization of the high molecular weight cytochrome *c* from *Desulfovibrio vulgaris* Hildenborough expressed in *Desulfovibrio desulfuricans* G200. *Biochemistry*. **31**: 3281–3288.
8. Pollock, W.B.R., M. Loutfi, M. Bruschi, B.J. Rapp-Giles, J.D. Wall, & G. Voordouw. (1991). Cloning, sequencing, and expression of the gene encoding the high-molecular-weight cytochrome *c* from *Desulfovibrio vulgaris* Hildenborough. *J. Bacteriol.* **173:** 220-228.
9. Moore, G.J., B. Pollock, R. Ganter, & K. Franklin. (1990). Evaluation of azidobenzoyl and benzoylbenzoyl groups for photoaffinity labelling of angiotensin receptors in responding tissues. *Proc. West. Pharmacol. Soc.* **33:** 261-264.
10. Pollock, W.B.R., P.J. Chemerika, M.E. Forrest, J.T. Beatty, & G. Voordouw. (1989). Expression of the gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) in *Escherichia coli*: export and processing of the apoprotein. *J. Gen. Microbiol.* **135:** 2319-2328.
11. Bisby, M.A. & B. Pollock. (1983). Increased regeneration rate in peripheral nerve axons following double lesions: enhancement of the conditioning lesion phenomenon. *J. Neurobiol.* **14:** 467-472.

Letters, notes, and communications:

1. Voordouw, G., W.B.R. Pollock, M. Bruschi, F. Guerlesquin, B.J. Rapp-Giles, & J.D. Wall. (1990). Functional expression of *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 in *Desulfovibrio desulfuricans* G200 after conjugational gene transfer from *Escherichia coli*. *J. Bacteriol.* **172:** 6122-6126.

Papers in conference proceedings:

1. Pollock, B., Beazer, M., Boothe, J., Keon, R., Moloney, M., Shafer, K. & Yang, H. (2006) Production and pilot scale recovery of bovine chymosin B from safflower seed. 232nd ACS National Meeting. September 10-14, 2006. San Francisco, U.S.A.

2. Yang, H., Shafer, K., Beazer, M., Pollock, B., Jickling, S., Szarka, S., Boothe, J., Bassuner, R., & Moloney, M. (2006). StratoCapture™ - Protein A – Novel immunoglobulin affinity purification technology with transgenic safflower oilbody in a continuous process. Recovery of Biological Products XII. April 2 - 7, 2006. Phoenix, U.S.A. Poster K6.
3. Pollock, B., Moloney, M. & Bassuner, R. (2004) Removal of host pigments from aqueous extracts of *Carthamus tinctorius* L. (Safflower) seed containing recombinant target protein. ISC '04. Oct. 2 – 8, 2004, Paris, France
4. Pollock, B., Beazer, M., Boothe, J., Shafer, K., Yang, H., Moloney, M. & Bassuner, R. (2003). *Carthamus tinctorius* L. (Safflower) seed as a new feedstock for manufacturing recombinant proteins. Recovery of Biological Products XI. Sept. 14 - 19, 2003. Banff, Canada. Oral Presentation in Workshop: New Recombinant Feedstocks & Techniques.
5. Hawrylechko, A., Dubord, P., Abdul-Wajid, A., Poole, S., Knaack, C., Jacobs, F., Young, M. & Pollock, B., (1999). Rational development of a second generation production process for recombinant Human Interleukin-2. Recovery of Biological Products IX. May 23 - 28, 1999. Whistler, Canada. Oral Presentation O9.3.
6. Pollock, W.B.R., Dumont, M. E., Rosell, F.I.& A.G. Mauk (1996). High-level expression in *Escherichia coli* of the J72K, C102T form of *iso*-1-holocytochrome *c* from *Saccharomyces cerevisiae*. Annual Meeting of the American Society for Biochemistry and Molecular Biology. June 2 - 6, 1996. New Orleans, U.S.A. *FASEB J.* **10:** Abstract #2235.
7. Voordouw, G., V. Nivière, & W.B.R. Pollock. (1991). Molecular biology of periplasmic hydrogenases and *c*-type cytochromes from *Desulfovibrio*. Abstract Third International Conference on Molecular Biology of Hydrogenases. July 29 - August 2, 1991. Troja, Portugal.
8. Pollock, W.B.R. & G. Voordouw. (1991). Analysis of the substrate specificity of the *Desulfovibrio* heme lyase system. 91st General Meeting of the American Society for Microbiology. May 5 - 9, 1991. Dallas, U.S.A. Abstract K45.
9. Loutfi, M., M. Bruschi, F. Guerlesquin, W.B.R. Pollock, & G. Voordouw. (1990). Characterization of a high molecular weight polyhemic cytochrome *c* (M_r 75 kDa) isolated from *D. vulgaris* Hildenborough. In "Control of charge transfer in cytochromes", Satellite Meeting of the International Congress of Biophysics in Vancouver. August 4 - 7, 1990. Montreal, Canada.
10. Voordouw, G., W.B.R. Pollock, M. Bruschi, F. Guerlesquin, B.J. Rapp-Giles, & J.D. Wall. (1990). Purification and characterization of *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 functionally expressed in *Desulfovibrio desulfuricans* G200 after conjugational gene transfer from *E. coli* . In "Control of charge transfer in cytochromes", Satellite Meeting of the International Congress of Biophysics in Vancouver. August 4 - 7, 1990. Montreal, Canada.
11. Pollock, B., G. Voordouw, & M. Bruschi. (1990). Cloning and sequencing of the gene encoding the high molecular weight *c*-type cytochrome from *Desulfovibrio vulgaris* Hildenborough. Annual Meeting of the Canadian Society for Microbiology. June 3 - 8, 1990. Calgary, Canada.
12. Pollock, W.B.R., P.J. Chemerika, & G. Voordouw. (1988). Expression of cytochrome c_3 from *Desulfovibrio vulgaris* in *Escherichia coli* . 17th Annual Meeting of the Western Branch of the Canadian Society of Microbiologists. Lake Louise, Canada.

Non-refereed abstracts:

1. Pollock, W.B.R. & G. Voordouw. (1990). Cloning, sequencing and expression of the gene encoding the high molecular weight cytochrome *c* from *Desulfovibrio vulgaris*. 10th Annual Meeting of the Alberta Heritage Foundation for Medical Research. Edmonton, Canada.

EXHIBIT B

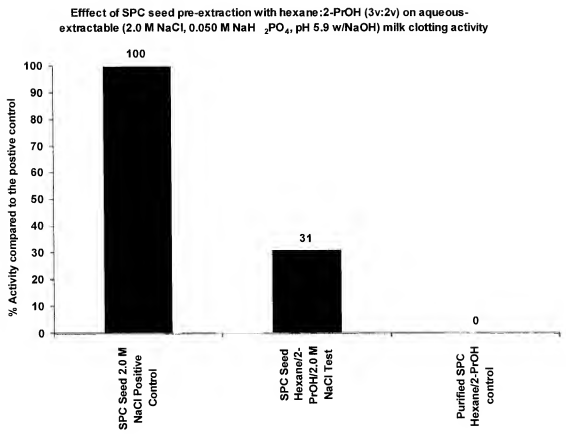


FIGURE 1

EXHIBIT C

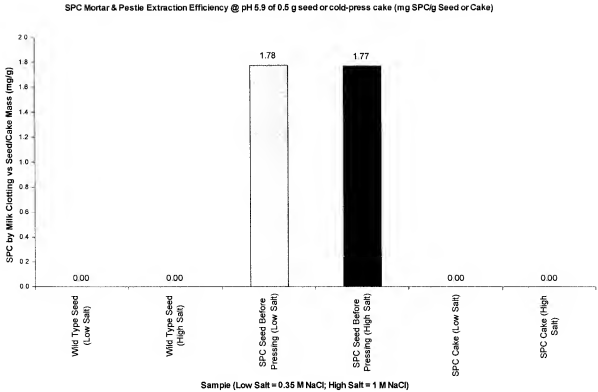


FIGURE 2

EXHIBIT D

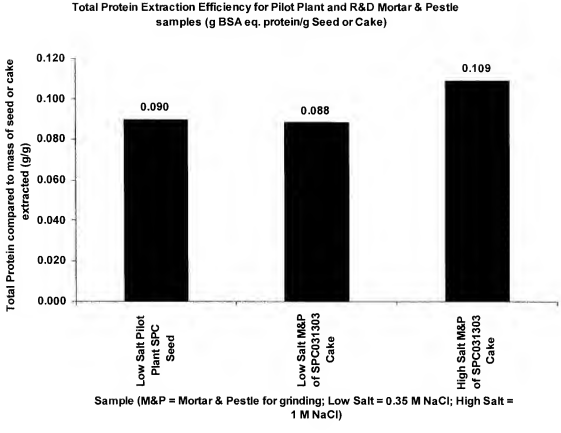


FIGURE 3